Introduction:

A community of organisms in aquatic ecosystem is dependent on each other and on their environment in which they live. The three main types of aquatic ecosystems are freshwater, marine, and estuarine ecosystems. The structure, function and performance of healthy and damaged aquatic ecosystem are understood by "Aquatic Ecosystem Health and Management". They focus on the development and application of management practices that will protect, maintain, remediate, or restore the health of these ecosystems and their sustainable use by human ecosystems. (Alexander *et al*, 1999) Aquatic ecosystems perform many important environmental functions. For example, they recycle nutrients, purify water, attenuate floods, recharge ground water and provide habitats for wildlife. (Loeb *et al*, 1994) Aquatic ecosystems are also used for human recreation, and are very important to the tourism industry, especially in coastal regions. (Daily *et al*, 1997) The health of an aquatic ecosystem is degraded when the ecosystem's ability to absorb a stress has been exceeded. A stress on an aquatic ecosystem can be a result of physical, chemical or biological alterations of the environment which are (1.) Physical alterations in water temperature, water flow and light availability. (2.) Chemical alterations in the loading rates of biostimulatory nutrients, oxygen consuming materials, and toxins. (3.) Biological alterations include the introduction of exotic species. Human populations can impose excessive stresses on aquatic ecosystems respectively. (Loeb *et al*, 1994). The organisms found in aquatic ecosystems are either autotrophic or heterotrophic namely *Chrococcales spp, Pleurocapsales spp, Ocillatoriales spp, Nostocales spp, Prochloron spp., Prochlorococcus spp., Prochlorothrix spp., Chloracidobacterium thermophilum, Rhodobacter spp., Rhodospirillum spp., Rhodopseudomonas palustris, Chlorobium spp., Chloroherpeton spp., Clathrochloris spp. Heliobacteria spp., Nitrobacter spp,, Nitrococcus spp,, Nitrospira spp., Nitrosomonas spp., Nitrosococcus, Nitrosolobus, Nitrosovibrio, Thiobacillus spp., Beggiatoa spp., Ferrobacillus spp., Gallionella spp. Hydrogenobacter spp. Bacillus spp., Clostridium spp., Sporohalobacter spp., Anaerobacter spp., Pseudomonas spp. Aeromonas spp., Neisseria spp., Streptococcus spp. Staphylococcus spp. Actinobacteria spp.* (Manahan *et al*, 2005)

Aeromonas spp. are common aquatic micro-organisms that occur in seawater, irrigation water, river water, brackish water, fresh water, ground water, spring water, industrial and domestic waste water. (Merich *et al*, 2011) The wide distribution of *Aeromonas* species in different aquatic ecosystems underlines their capacity to adopt with environments in different tropics levels. Several studies have shown that the phenospecies *Aeromonas hydrophila* is prevalent in cleaner water e.g. rain water, whereas *Aeromonas caviae* is prevalent in water with a high level of fecal pollution, the frequencies of the other phenospecies appear to be unrelated to environmental conditions. But few studies show

that the count of *Aeromonas* species are usually found more during summer than in winter. (Burke *et al*, 1984)

There are 22 different species of *Aeromonas* known till date. There are eleven named speices and their subspecies commonly found in aquatic environment. viz. *A.allosaccharophila, A.schubertii, A.sorbia, A.veronii, (A.veronii subsp. sorbia) A.media, A.hydrophila, A. salmonicida subsp. salmonicida, (A. salmonicida subsp. smithia, A. salmonicida subsp. masoucida, A. salmonicida subsp. achromogenes) A.trota, A.eucrenophila, A .jandaei, A. caviae.* Different species of *Aeromonas* such as., *A.hydrophila, A.caviae ,A.veronii ,A.schubertii, A.jandaei* and *A.trota* are associated with diarrheal illness and can cause infections and septicemia. The infections can occur in healthy people of all age and are often acquired through foreign travel. Wound infections are relatively uncommon but can progress rapidly if not treated. The main species causing of human infection are *A.hydrophila ,A.caviae, A.veronii A.media* and *A.veronii subsp. sorbia*. Not only in human being, some of them also causes infections in fish. Some motile species are associated with hemorrhagic septicemia in freshwater fish. *A.hydrophila, A.caviae* and *A.sorbia,* are pathogenic for fish and amphibians. (Macfadin 2000, Martin *et al*, 1996)

The *Aeromonas* species belong to class gammaproteobacteria, family: Aeromonadaceae, kingdom: Bacteria, order: Aeromonadales, phylum: Proteobacteria genus: Aeromonas, subdivision : *A. veronii, A. sobria, A. schubertii, A. jandaei, A. hydrophila, A. encheleia, A. bestiarum, A. ichthiosmia, A. enteropelogenes , A. bivalvium, A. allosaccharophila, A. simiae, A. sharmana, A. Salmonicida, A. punctata, A. popoffii, A. molluscorum, A. media, A. euchrenophila*. (Brenner 2005)

Aeromonas species are gram negative, motile, facultative anaerobic, rod shaped, Oxidase positive bacteria of the recently assigned family *Aeromonadaceae*. (Brenner 2005, Macfadin 2000) The significance of *Aeromonas* species as causative agent of human diarrhea has recently been established. (Eze *et al*, 2011, Jennifer *et al*, 2006) *Aeromonas* species have been found to cause a variety of primary infections in the normal host as well as severe infections in immune-comprosied patients. The *Aeromonas* species from the heterogenous environment possed several virulence factors such as enterotoxins, haemolysins, cytotoxins, proteases, adhesions etc. (Brenner 2005, Eze *et al*, 2011, Jennifer *et al*, 2006, Khalifa *et al*, 2000, Martin *et al*, 1996)

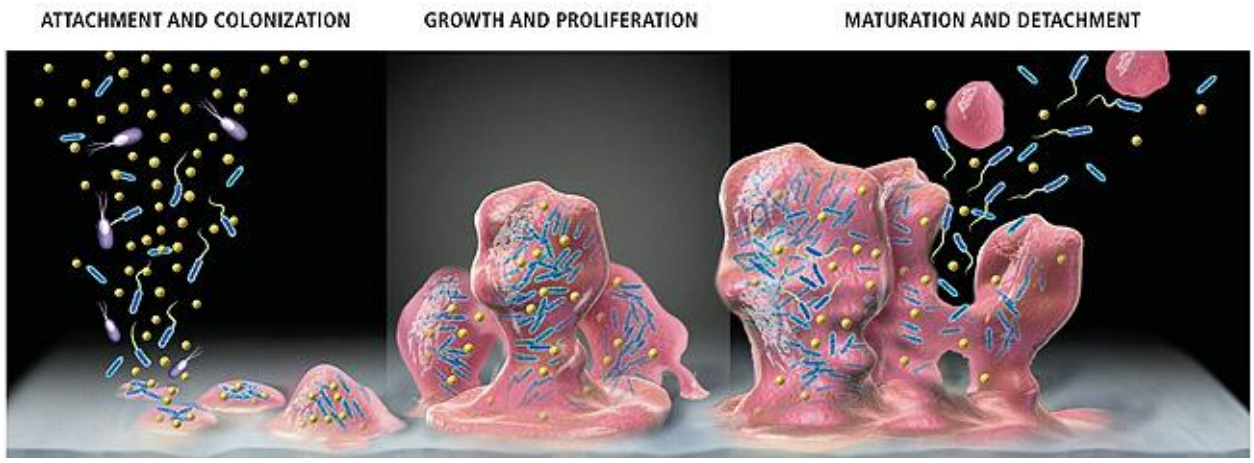
These bacteria have also been isolated from chlorinated and unchlorinated drinking water and from bottled mineral water, which shows that they are able to withstand long periods of nutrient limitation. For this reason, the potential public health significance cannot be ignored. The growing interest in these organisms addresses aquatic environments. As aquatic environments represents an important reservoir of *Aeromonas* species. It assumed that human infections arise from recreational activities,

contaminated drinking water and sea foods. (Maria *et al*, 2000, Marisa *et al*, 2007) The importance of water for survival and diffusion of the micro-organisms requires evaluating the different physical and chemical characteristics and aquatic Biofilm, which are well-organized communities of microorganisms, are widespread in nature. In aquatic ecosystem groups of microorganisms cooperatively from Biofilm on the various support system may be pipe line, fishes, aquatic plant etc. Microorganisms remain in the aquatic system either as a suspended form or as a Biofilm. (Sylvia *et al*, 2004)

A biofilm is an aggregate of microorganisms in which cells adhere to each other on a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). Biofilm EPS, which is also referred to as slime (although not everything described as slime is a biofilm), is a polymeric conglomeration generally composed of extracellular DNA, proteins, and polysaccharides. Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial and hospital settings. (Hall-Stoodley *et al*, 2004, Lear *et al*, 2011) Microbes form a biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases, by exposure of planktonic cells to sub-inhibitory concentrations of antibiotics. (Karatan *et al*, 2009, Jenifer *et al*, 2006) When a cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in behavior in which large suites of genes are differentially regulated. (An D *et al*, 2007) Formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. These first colonists adhere to the surface initially through weak, reversible adhesion via. Van Der Waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili. (Kaplan *et al*, 2003) The first colonists organism facilitate the arrival of other cells by providing more diverse adhesion sites and beginning to build the matrix that holds the biofilm together. Some species are not able to attach to a surface on their own but are often able to anchor themselves to the matrix or directly to earlier colonists. It is during this colonization that the cells are able to communicate via quorum sensing using such products as N-Acylhomoserine lactones (AHL). Once colonization has begun, the biofilm grows through a combination of cell division and recruitment. The final stage of biofilm formation is known as development, and is the stage in which the biofilm is established and may only change in shape and size. The development of a biofilm may allow for an aggregate cell colony (or colonies) to be increasingly antibiotic resistant. With water disinfection in distribution systems both organic and inorganic nutrients to biofilm development and the subsequent impact of developed biofilms on disinfectant decay are important requirements for distribution system management strategies. Biofilms are usually found on solid substrates submerged in or exposed to an aqueous solution,

although they can form as floating mats on liquid surfaces and also on the surface of leaves, particularly in high humidity climates. Given sufficient resources for growth, a biofilm will quickly grow to be macroscopic (visible to the naked eye). Biofilms can contain many different types of microorganism, e.g. bacteria, archaea, protozoa, fungi and algae; each group performs specialized metabolic functions. However, some organisms will form single-species films under certain conditions. Bacteria such as *Pseudomonas aeruginosa, Aeromonas species* etc. have genes that are turned on in about 15 mins after the attachment to a surface. One gene is algC and is needed to make alginate - one of the components of the polysaccharide matrix material. *Aeromonas* species are capsulated and capable to produce exoploysaccaride which support in formation of Biofilm.

Mechanism of Biofilm formation.



Many biofilm bacterial cells typically make dozens or hundreds of proteins not found in "free-floating" cells. The cells signal to each other as the approach the "quorum" or number required to initiate biofilm formation. It seems as if a certain number of cells are needed to produce enough of the signal molecules to "switch over" the cells to matrix production - this is the "quorum". In *Aeromonas species* and similar cells, the signal molecule is known - they are acylated homoserine lactones. If the genes for these compounds are missing - no biofilms are formed. Research indicates that multispecies biofilms are generally more resistant to disinfection and different antibiotics than single-species biofilms. Biofilm in aquatic system is necessary to study as water is the universal source for all living creatures. As mention earlier that organisms present in the Biofilm may cause different diseases to human beings, animals, amphibians and other aquatic animals like fish, frog etc. As fish is the staple diet of many, it can lead to spread of infection to humans. (Eze *et al*, 2011, Hall-Stoodley *et al*, 2004, Sylvia *et al*, 2004)

Several virulence factors have been identified in *Aeromonas spp*. The heat-labile and stable cytotoxin that has enterotoxic activities, together to the ability to produce hemolysins and proteases is a relevant attribute of pathogenic *Aeromonas spp*, as the production of adhesions. The ability of aeromonads to adhere to the human epithelial cell model has been correlated with the enteropathogenicity of the genus. (Denise et al, 2008; Kaplan et al, 2003; Fiorentini et al, 1998; Kaper et al, 1981) More recent studies investigating the adherence of *A. caviae* to HEp-2 cells suggested the involvement of the polar flagellum in this process. Many microorganisms secrete enzymes which ensure their penetration into the host cells. Phospholipases belong to this type of molecules capable to damage or destroy cell surface membranes. Within the large family of lipolytic enzymes, phospholipases constitute a very diverse subgroup with physiological functions such as digestion and signal transduction. Most phospholipases may associate with membranes at the lipid-water interface. Because of *Aeromonas hydrophila's* structure, it is very toxic to many organisms. When it enters the body of its victim, it travels through the bloodstream to the first available organ. It produces Aerolysin Cytotoxic Enterotoxin (ACT), a toxin that can cause tissue damage. *Aeromonas hydrophila, Aeromonas caviae*, and *Aeromonas sobria* are all considered to be opportunistic pathogens, meaning they rarely infect healthy individuals. (Janda et al, 1998; Borrego et al, 1991, Isoken et al, 2012)

Aeromonas hydrophila is widely considered a major fish and amphibian pathogen, and its pathogenicity in humans has been recognized for decades. It was believed that the pathogenicity of *Aeromonas* spp. is mediated by a number of extracellular proteins such as aerolysin, hemolysins, enterotoxins, lipase, chitinase, amylase and gelatinase. The detection of virulence factors of Aeromonas hydrophila is a key component in determining potential pathogenicity because these factors act multifunctionally. Aerolysin, a toxin that can cause tissue damage and target surface of eukaryotic red blood cells and forms pores in the cell membrane causing lysis. For rapid detection of virulence factors of isolated Aeromonas spp., a polymerase chain reaction assay can be use. (Denise et al 2008).

However the pathogenic mechanisms of *Aeromonas* spp. is remain unknown. The recently proposed type III secretion system (TTSS) mediated pathogenic mechanism has been proven to play role in *Aeromonas* pathogenesis. The TTSS is specialized protein secretion machinery that export virulence factors delivered directly to host cells. These factors disturb normal host cell functions in ways that are beneficial to invading bacteria. In contrast, type III secretion system is triggered when a pathogen comes in contact with host cells. ADP-ribosylation toxin is one of the effector molecules secreted by several pathogenic bacteria and translocated through TTSS and delivered into the host cytoplasm leads to cytoskeletal damage and apoptosis. This toxin has been characterized in *Aeromonas hydrophila* (human diarrhoeal isolate) (Isoken et al 2012).

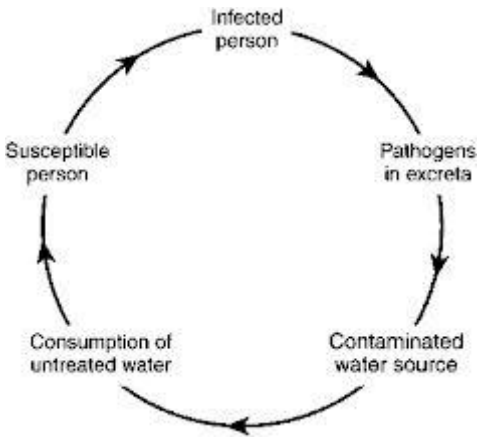
Aeromonas hydrophila causes a variety of diseases in both fish and human populations. *Aeromonas hydrophila* is very toxic to many organisms because of its structure. When it enters the body of fish, amphibians, or humans, it travels via the bloodstream to the first available organ. It produces aerolysin cytotoxic enterotoxin (ACE) which is one of the major virulence factors. Its toxin is produced and secreted by the cell from a type II secretion system. The toxin binds to high- affinity receptors and undergoes oligomerization to form a heptameric pore- forming complex which allows passage of small molecules in the plasma membrane, resulting in permeabilization of the cell, cell death, and eventually tissue destruction. *Aeromonas hydrophila* is also known as an opportunistic pathogenic bacterium, meaning they only infect hosts with weakened immune responses. (Albert et al 2000, Chopra et al 2000). Other virulence functions include a surface layer which inhibits complement- mediated killing, type IV pili for attachment, and a set of extracellular proteases which can cause tissue damage. (Seshadri et al, 2006).

Aeromonas species have increased in recent years since they cause gastroenteritis, cellulitis, peritonitis, meningitis, and pneumonia in immunocompromised humans and disseminated infections in immunocompromised hosts (Von Graevenitz, et al 2007). In developing countries, *A. hydrophila* is also known to cause diarrhea in children (Dallal et al 2004) and travelers (Jiang et al 2002). Known risk factors that predispose humans to the disease include drinking or swimming in contaminated water and also ingestion of contaminated food.

One of the diseases *Aeromonas hydrophila* can cause in humans is gastroenteritis. This disease can affect anyone, but it occurs most in young children and people who have compromised immune systems or growth problems. This bacterium is linked to two types of gastroenteritis. (Ormen et al, 2001; Vadivelu et al, 1995)The first type is a disease similar to cholera, which causes rice-water diarrhea. The other type of disease is dysenteric gastroenteritis, which causes loose stools filled with blood and mucus. Dysenteric gastroenteritis is the most severe out of the two types, and can last for multiple weeks. *Aeromonas hydrophila* is also associated with cellulitis, an infection that causes inflammation in the skin tissue. It also causes diseases such as myonecrosis and eczema in people with compromised immune systems. Though *Aeromonas hydrophila* can cause serious diseases, there have never been serious outbreaks.

Aeromonas species are found globally in surface water, ground water, chlorinated drinking water, nonchlorinated drinking water, bottled mineral water (Villari et al 2003, Chauret et al 2001 and Taweel et al 2001) and broad range of foods (McMahon et al 2001). Thus Aeromonas spp. can

prevalent in water, which is the basic necessity of humans. Thus transmission may occur from fish to water to human through water.



Aeromonas species are known as causative agents of a wide spectrum of diseases in man and animals (Ghenghesh et al, 2008). Some studies have shown that some motile *Aeromonas* species are becoming food and waterborne pathogens of increasing importance (Araujo et al, 2002 and Ansari et al, 2011). They have been associated with several food-borne outbreaks and are progressively being isolated from patients with traveler's diarrhea (Von Graevenitz, et al 2007). Presently, as a putatively emerging enteric pathogen, *Aeromonas* species have the inherent capability to grow in water distribution systems, especially in biofilms, where they may be resistant to chlorination (Chauret et al 2001).

Aeromonas species have been found in chlorinated drinking water supplies in several countries (Fernandez et al, 2000 and Figueras et al, 2005) and typically of densities less than 10 cfu/mL in drinking water distribution systems. They occur in distribution system biofilms where they may be protected from disinfection (Chauret et al 2001) and multiple strains are frequently found in water sources (Sen et al, 2004). Knowing the effects of disinfectant on bacterial growth helps to understand how to prevent and treat infections. Not all disinfectants work the same. One of the most important chemicals in use today is disinfectant is chlorine. Some do not kill bacteria but merely stop their growth. Certain common disinfectants sold in stores are ineffective at killing germs and pose a health risk. Several different types of disinfectants exist with varying effects on bacterial growth, according to the National Human Health. The typical disinfectant stops the growth of bacteria by killing them. A disinfectant cannot kill every single bacterial microbe. Some bacteria have a random mutation that prevents toxicity to the disinfectant. These bacteria survive and produce new populations resistant to the specific disinfectant. For prevention and treatment for *Aeromonas* outbreaks, every attempt should be made to identify and eliminate sources of environmental stress. This alone will often

correct the disease problem. (Stanely *et al*, 1982, Tadashi *et al*, 1995) Avoid handling fish when they are in a weakened state or when environmental conditions are less than optimal. Fish should never be handled or transported during an *Aeromonas* outbreak. The increasing use of granulated activated carbon in water treatment may allow proliferation and dissemination of *Aeromonas.* Limiting the numbers of Aeromondas released into distribution systems thus requires affective management of filter beds and maintenance of adequate final chlorination.

Bacterial adherence to the epithelial surface is considered as one of the important factors in the infection process. Adherence is an interaction between adhesion molecules on the bacterial cell surface and complementary receptor molecules on the host cell surface. Microorganisms adhere to epithelial cells in a highly selective manner and thus cannot be removed by unspecific defense mechanisms of the urinary, respiratory, gastrointestinal, and genital tracts. Microbial adhesion to hydrocarbons is a biphasic separation hydrophobicity assay which measures relative CSH through the capacity of the cell to adhere to a hydrocarbon phase. Employing the CHS classification scheme described by Schneider and Riley (1991), isolates with % hydrophobicity > 70% were classified as highly hydrophobic and those with a hydrophobicity index < 30% were classified as highly hydrophilic. The hydrophobic properties of bacteria are responsible for formation of biofilm and adhesion to epithelial cells. (Denise et al, 2008)

The prevention of the spread of *Aeromonas* strains involved in diarrhea depends on ensuring appropriate sanitary measure like proper food preparation, hand-washing, and efficient sewage disposal system. Proper surveillance of water, food, and sanitation facilities, using public health diagnostic and detection procedures is essential to protect individuals including infants from *Aeromonas* strain diarrhea. In the aquaculture environment, management consists of disposal of diseased animals and water treatment to control the densities of aeromonads in aquaculture systems. Maintaining high standards of water quality, temperature control, disinfection of equipment, and stress reduction reduce the prevalence of disease. In aquaculture system the quality of the water is of paramount importance in the prevention of contamination of fish or plants grown in wastewater ponds. Reliance has been placed principally on minimizing the risk of pathogen transmission by thorough cooking of the products, but this approach has not always been satisfactory and, where the pond products are eaten uncooked, no health protection is provided. Therefore, the presence of potentially pathogenic and multidrug-resistant *Aeromonas* strains in surface waters remains of significant health implication. Continuous monitoring of surface waters is vital to identify potential water-borne pathogens and to reduce the health risk caused by the Aeromonads. (Isoken et al, 2012).

Aims:

- Isolation of target organism from water samples.
- To study phenotypic characterization and Molecular characterization of isolates.
- To determine Antimicrobial susceptibility pattern.
- To study Enzyme profile of the isolated organisms
- To check Chlorine sensitivity
- To study Hydrophobicity assay of the isolates
- To check for Biofilm formation on polystyrene micro-titre plate by the isolates.
- To Detect of Aerolysin gene (aer gene) using PCR.

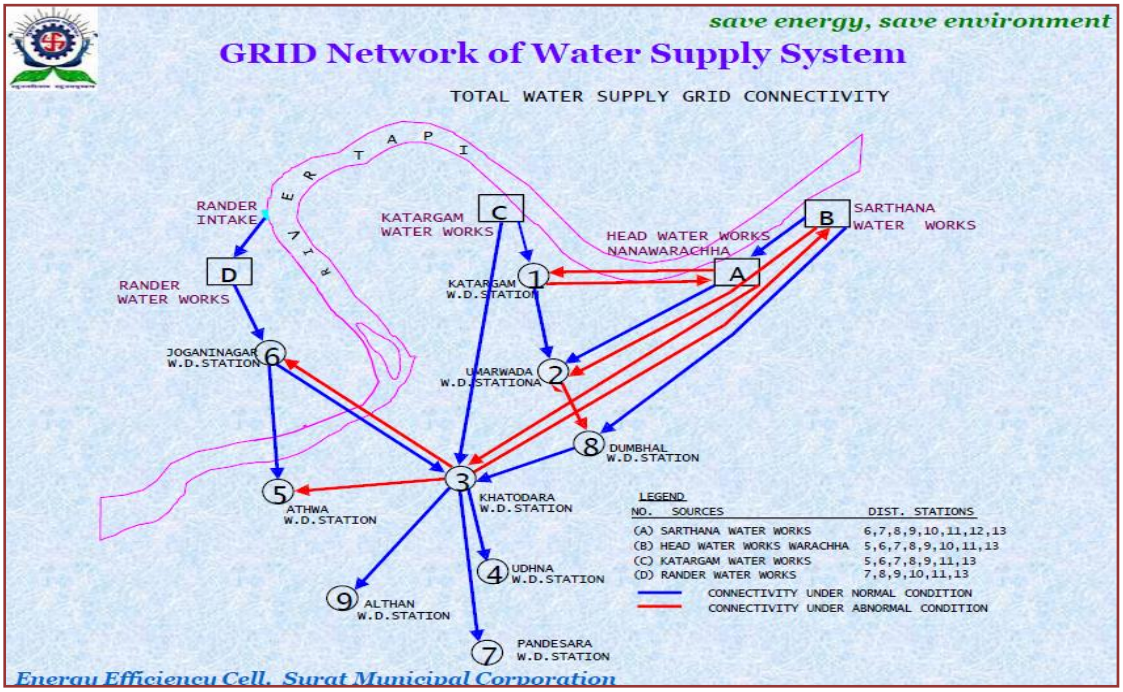
Objectives:

- This will ultimately serve to check the quality of water and water distribution system.
- Finally it will help to prevent health hazards and serve the purpose of public health.
- *Aeromonads, Campylobacter jejuni, Yersinia enterocolitica, Mycobacterium spp., Legionella pneumophila* and multidrug-resistant *Pseudomonas aeruginosa* have been associated with water-borne illnesses which are new organisms in water standards.(Emerging issues in water and infectious diseases-Basic information about pathogen and indicator in water, 2013) and (Micobial fact sheets- WHO, 2007)

Materials and Methods:

Sampling:

Every month samples were collected from four main water works, nine different pumping station as shown below and different domestic water samples from June 2013 to May 2014.



Here three seasons were considered viz. June 2013 to September 2013-monsoon season, October 2013 to January 2014-winter and February 2014 to May 2014-summer season. As *Aeromonas spp*. are found in drinking water; the samples need to be collected from the similar sites every month to check seasonal variations.

The water samples are collected from different sites as mention in the.

WATER WORKS:	
WW1	NANAWARACHHA
WW2	SARTHANA
WW3	KATARGAM
WW4	RANDER
PUMPING STATION:	
WPS1	KATARGAM
WPS2	UMARWADA
WPS3	KHATODARA
WPS4	UDHNA
WPS5	ATHWA
WPS6	JOGANINAGAR
WPS7	PANDESARA
WPS8	DUMBHAL
WPS9	ALTHAN
T1	SMC tap water
DOMESTIC WATER SAMPLES:	
PB1	AQUA GUARD CRYSTAL WATER PURIFIER (power boiling +)
R.O.	AQUA GUARD REVIVA WATER PURIFIER
U.V	AQUA GUARD CLASSIC WATER PURIFIER
R.O. + U.V.	AQUA GUARD DUO WATER PURIFIER

Isolation

The selective isolation was carried out by using Membrane Filter Technique (0.45 μm) to get enumerate *Aeromonas* in drinking water by using M-*Aeromonas* selective media, incubated at 30^0 C (Burke V *et al* 1984; Sharon *et al* 2003). 100 ml of water sample was filtered for enumeration. Isolated colony is selected for further screening. For selective isolation of *Aeromonas hydrophila*, Rippey Cabelli agar plate (Hi media, Mumbai) was used at 30^0 C. (Rippey *et al* 1980).

Phenotypic Identification

The morphological and colony characteristics were studied using Nutrient agar plate and Mac Conkey's agar plate along with highly selective medias M-*Aeromonas* selective media and Rippey Cabelli agar plate. The physiological characteristics of all the obtained isolates were studied. The biochemical characteristics (Oxidase, Catalase, Indole, Methyl Red, V-P Test, Citrate (Simmons), H_2S Production , Brown Soluble Pigment, Gelatin, Urea, Lipase, Bile esculin, Lysine Decarboxylase,

Nitrate, Phenylalaine, and various Sugars) were also carried out from them, using standard references. (Brenner et al 2005; Macfadin 2000; WHO 2007.)

Antimicrobial susceptibility

All bacterial species were further tested for their antimicrobial susceptibility towards the different groups of antibiotics using standard disk with known concentration. The antibiotic used for these purpose were listed below along with their concentrations. (Denise *et al*, 2008, Jennifer *et al*, 2006, Khalifa *et al*, 2000). Antibiotic susceptibility test were performed as Kirby-Bauer disc diffusion method. This method is recommended by the National Committee for Clinical Laboratory Standards, now known as Clinical and Laboratory Standards Institute (C.L.S.I.) and the World Health Organization (W.H.O.). The results were interpreted as per the guidelines provided along with the susceptibility disc. The results were compared with the standards shown below.

Different antibiotics use for the study.

Antibiotics	**Concentration of Antibiotics (µg)**	**Observation & Interpretation (Inhibition zone diameter in mm)**		
		Resistant	**Intermediate**	**sensitive**
β- lactum				
Imipenem	10	13	14-15	16
Meropenem	10	13	14-15	16
Ampicillin	10	13	14-15	16
Ceftriaxone	30	13	14-20	21
Cefotaxime	30	14	15-22	23
Ceftazidime	30	14	15-17	18
Cefazoline	30	14	15-17	18
Cefpirome	30	14	15-17	18
Cefipime	30	14	15-17	18
Aminoglycosides				
Amikacin	30	14	15-17	18
Gentamycin	10	12	13-14	15
Netromycin	30	12	13-14	15
Kanamycin	30	13	14-17	18
Quinolones				
Ciprofloxacin	5	15	16-20	21
Clindamycin	2	14	15-20	21
Glycopeptides				
Vancomycin	30	14	15-16	17
Extended spectrum				
Amoxy + Clavulanate	30	13	14-17	18
Cefotaxime + Sulbactum	30/15	14	15-17	18
Piperacilline + Tazobactum	100	17	18-20	21

Misseliansces				
Nalidix acid	30	13	14-18	19
Chloramphenicol	25	12	13-17	18

Hemolysis, phospholipase, and DNase production

Hemolytic assays were done by observing the zone of hemolysis around colonies on a sheep blood agar plate as described in an earlier report (Imzilm et al 1996) DNase agar (Dnase test agar, Hi Media, Mumbai) change in blue to pink or purple or a zone of clearing around the bacterial colonies indicated a positive test. The phospholipase production was carried out, with some modifications,as previously described (Polak 1992; Price et al 1982). The test medium consisted of MA containing 1 M sodium chloride, 0.005 M calcium chloride, add 2% egg yolk. Each Aeromonas isolated was inoculated. After incubation at 37°C for 3–8 days, the diameters of the colonies and dense opaque zones around the growth indicated positive test.

Biofilm formation

To check for Biofilm formation, polystyrene micro titer plate was used. The wells were filled with M-Aeromonas selective medium which will allow the growth of only *Aeromonas* spp. Wells were labeled and were inoculate with the respectively isolates. After different time intervals like1hr, 2hrs, 3hrs, 4hrs, 5hrs, 6hrs was considered, and excess medium was drain and SPC was done using Nutrient Agar medium from the well by scraping along with antimicrobial susceptibility from biofilm. (Oliveira *et al* 2003).

Hydrophobicity assay

For the hydrophobicity assay, each isolate of Aeromonas was inoculated into 5 ml of Trypticase soy broth for 16–18 h at 37°C. The bacteria were then harvested by centrifugation, washed twice, and suspended in 5 ml of 50 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl to an absorbency of 0.4 at 660 nm. The bacterial suspension was treated with xylene (2.5:1 v/v; Merck). Following 20 min pre-incubation at room temperature, the mixture was vigorously mixed for 2 min. After allowing 20 min for the hydrocarbon phase to rise completely, the aqueous phase was carefully removed with a Pasteur pipette. The absorbancy was then determined at 660 nm against a blank consisting of sodium phosphate buffer. The cellular superficial hydrophobicity (CSH) was calculated as previously described (Rosenberg *et al* 1980).

Chlorine sensitivity test

The bactericidal effect of chlorine on Aeromonas isolates was determined using the method of Seyfried and Fraser (1980), with the following modification. In150 ml flask, 50ml chlorine free water

was taken and appropriate chlorine solution was added like, concentration: 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, mg/l. Add 10 ml. bacterial suspension. Mix properly and allow 1 min contact period. 5 ml (0.7 mg/ml.) Sodium thiosulfate solution was added to neutralize the chlorine and thereby stop the reaction. Remove the content and plate on Nutrient Agar plate. Plates were counted after 48 h of incubation at 37°C.

Isolation of DNA

The isolation of DNA was done using spin column method, the kit for DNA isolation was being proceed from the standard company (Himedia), and the protocols was followed as per their instructions.

Detection of *Aero* gene by PCR

The polymerase chain reaction (PCR) was done using appropriate primer from Xcelris genomics and standard kit for PCR from (Bangalore genei) and protocols was followed as per their instructions. The polymerase chain reaction (PCR) was used to detect the presence of the aerolysin in all isolates. The primers used were Aero 2F: 5'-AGC GGC AGA GCC CGT CTA TCC A-3' and Aero 2R: 5'-AGT TGG TGG CGG TGT CGT AGC G-3'. PCR was carried out on a cycler using the following cycle: preheating at 95°C for 5 min followed by 30 cycles at 95°C for 2 min, 55°C for 1 min and 72°C for 1 min, followed by 7 min final extension at 72°C. PCR products were examined by electrophoresis in 1% agarose gel in TBE buffer. The gel was stained with EtBr and saw under UV light (Yogananth *et al* 2009; Niamah *et al* 2012).

Molecular characterization of isolates

The isolates showing presence of aero gene were further identified by their Molecular characterization by 16S rDNA. (Xcelris genomics- Ahembabad)

Results and Discussion:

All water samples were analyzed microbiologically using standard methods mentioned in APHA & WHO/FAO standards for water (Clesceri LS et al, 1998). The selective enumeration of *Aeromonas* species was carried out by using membrane Filter Technique using M-*Aeromonas* selective media. (Burke V *et al* 1984; Sharon *et al* 2003).

Table 1: Enumeration of *Aeromonas* species.

Samples	June J1	July J2	August A1	September S	October O	November N	December D	January J3	February F	March MI	April A2	May M2
WW1	25	23	20	18	17	09	08	05	no growth	23	26	33

WW2	17	18	18	15	12	11	13	11	08	16	14	19
WW3	15	13	33	12	10	12	16	15	no growth	11	13	16
WW4	25	29	09	33	24	20	19	02	19	no growth	32	36
WPS1	07	no growth	24	14	04	09	no growth	09	no growth	no growth	no growth	no growth
WPS2	36	34	21	no growth	12	19	22	17	no growth	34	no growth	40
WPS3	66	50	40	60	40	22	50	22	no growth	31	50	66
WPS4	30	28	56	48	30	21	41	20	13	32	29	33
WPS5	no growth	no growth	32	20	no growth	12	18	10	no growth	12	30	32
WPS6	no growth	no growth	16	11	no growth	11	11	no growth	11	20	15	12
WPS7	35	37	09	07	02	no growth	30	no growth	15	33	39	45
WPS8	20	24	25	14	19	08	22	no growth	20	09	20	24
WPS9	no growth	no growth	15	10	09	08	17	no growth	17	18	18	17
T1	uncountable	132	uncountable	120	98	Uncountable	90	uncountable	77	210	187	Uncountable
PB1	10	12	10	07	09	08	10	06	13	12	11	19
RO	07	09	22	02	no growth	07	07	05	05	07	09	05
UV	no growth	no growth	07	06	no growth	04	no growth	04	no growth	07	no growth	no growth
RO+ UV	no growth	no growth	05	09	no growth	02	no growth	05	no growth	09	no growth	no growth

The young colonies were bright yellow color which turns to greenish hue on aging. The total numbers of isolated isolates are 199, out of which mostly all of them showed smooth, small, circular and with no pigmentation except 11 isolates which showed brown pigmented colony. The highly selective media is Rippey Cabelli which was used for *Aeromonas hydrophila*, the most pathogenic bacteria among all *Aeromonas* spp. which gives blue color colony of *Aeromonas hydrophila.* (Fig. 1). Total 96 isolates was of *Aeromonas hydrophila* from 199.

Figure: 1: Colony characteristics of isolates on different medium.

The total number of isolates was counted and further proceeded for their morphological and motility test. The colony characteristics of the obtained isolates were studied using Nutrient agar plate and Mac Conkey's agar plates, which were observed as small pin point colonies and lactose fermenter colonies respectively. Considering the morphological studies they were Gram negative, short rods, appeared singly and 188 were motile, while 11 isolates which showed brown pigmented colony on Nutrient agar plate were non motile.

Along with these of they were identified on the basis of their biochemical characteristics also. Considering all phenotypic characteristics, they were identified as *Aeromonas hydrophila* (96), *Aeromonas sobria* (26), *Aeromonas caviae* (25), *Aeromonas Salmonicida subsp. Salmonicida* (11), *Aeromonas schubertii* (12), *Aeromonas veronii* (12), *Aeromonas euccrenophila* (08), *Aeromonas trota* (05) *and Aeromonas popoffii* (04).

The monthly count of Aeromonas is considered for three seasons i.e. Monsoon (June 2013 to September 2013), winter (October 2013 to January 2014) and summer (February 2014 to may 2014). Our seasonal variations study shows that the count was often more during the warmer months.

As *Aeromonas* spp. is found in aquatic environment as well as pathogens to immune suppressed individuals, fish, frogs and other amphibians. As fish is consumed by humans, the disease can spread form fish to humans. The Aeromonas spp. is also capable of forming Biofilm, so it may form layer or film on the membrane of intestine. Considering their pathogenicity the antibiotic susceptibility testing was also carried out and majority of Aeromonas species under study were found to be susceptible to broad spectrum antibiotics such as Ciprofloxacin, Piperacililline + Tazobactum and Gentamicin. But majority were resistant to Ampicillin, Chloramphenicol and Ceftriaxone.x

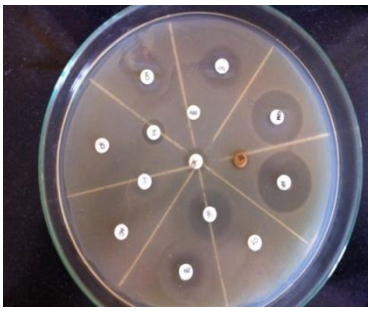


Figure: 2: Result of Antibiotic susceptibility test.

Different Enzyme profile was done of the isolates which is also one of the virulence factors of *Aeromonas spp.* The results of present study indicate the difference in the expression of DNase and phospholipase by Aeromonas spp. isolates. All isolates showed β-haemolysis.

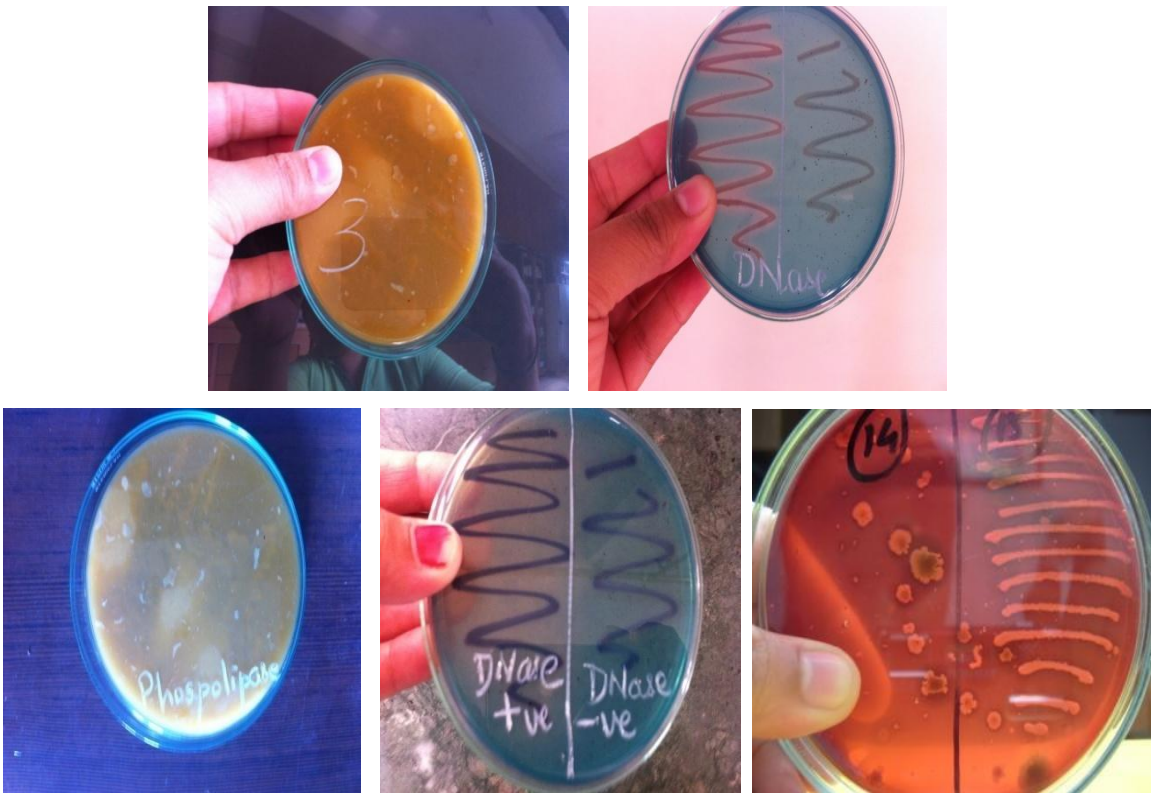


Figure 3: Haemolysin, Dnase and Phospholipase enzyme.

Aeromonads has capsulated rods and so colonize with other organisms to form biofilms in water distribution and food-processing systems as well as on water-dwelling plants and animals (e.g., fish, leeches, and frogs). The organisms grew well in the medium, reaching a maximum number of counts. The number of adherent bacteria was maximum at 4 h. Thus it proves that Aeromonads are efficient colonizers of surfaces and are an important constituent of bacterial biofilms in both water distribution systems and food processing environments. Biofilm formation in *Aeromonas hydrophila* and *Aeromonas veronii* showed maximum biofilm formation within 4 hours. When Antibiotic suseptibility on biofilm formation was done and majority of *Aeromonas* species under study were found to be susceptible to broad spectrum antibiotics such as Ciprofloxacin, Piperacililline + Tazobactum and Kanamycin. But majority were resistant to ampicillin, Chloramphenicol and Nalidix acid. The comparititive study of antibiotic susceptibility testing and Antibiotic suseptibility on biofilm formation was done, a change was found in broad spectrum antibiotics Gentamicin and Kanamycin, while a change was also found in resistant pattern to Nalidix acid and Ceftriaxone respectively.

Bacterial adherence to the epithelial surface is considered as one of the important factors in the infection process. Adherence is an interaction between adhesion molecules on the bacterial cell surface and complementary receptor molecules on the host cell surface. Microorganisms adhere to epithelial cells in a highly selective manner and thus cannot be removed by unspecific defense mechanisms of the urinary, respiratory, gastrointestinal, and genital tracts. Microbial adhesion to

hydrocarbons is a biphasic separation hydrophobicity assay which measures relative CSH through the capacity of the cell to adhere to a hydrocarbon phase. Employing the CHS classification scheme described by Schneider and Riley (1991), isolates with % hydrophobicity > 70% were classified as highly hydrophobic and those with a hydrophobicity index < 30% were classified as highly hydrophilic. The hydrophobic properties of bacteria are responsible for formation of biofilm and adhesion to epithelial cells. *A. hydrophila, A. sobria* and *A. caviae* showed < 30% hydrophobicity so they are highly hydrophilic in nature. Whereas *A. euccrenophila* and *A. trota* showed hydrophobic nature. In the current study, the presence of residual chlorine, as in the case for many other bacteria, does not necessarily exclude the Aeromonas isolates. *A. hydrophila* and *A. sobria* showed highest chlorine tolerance even at 0.2 mg/ml to 0.5 mg/ml. Whereas *A. euccrenophila, A.popoffii* and *A. trota* showed less chlorine tolerance. Similar tolerance to chlorine was seen in all strains regardless of source of isolation. This is particularly noteworthy because this chlorine concentration is six times the recommended level of ≥0.2 mg free chlorine per liter. Whether the higher tolerance to chlorine of Aeromonas isolates can be linked to greater virulence is unclear.

Detection of Aero gene was done using gel electrophoresis; the bands appearance in the amplified *Aero* gene of isolates from water samples shows the molecular weight of aerolysin (*Aero* gene 424 bp). Amplification result obtained through the present study also shows the same molecular weight of aerolysin gene.

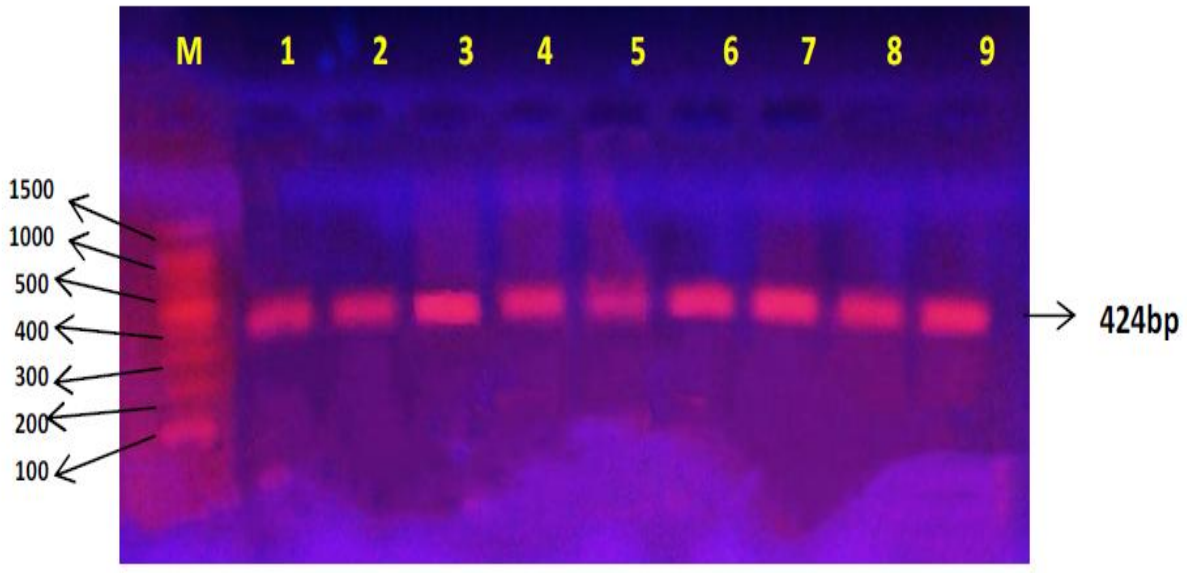


Figure 4: Electrophoresis of PCR production on 1% agarose gel, Lane 1 is marker DNA standard (100-1500) bp and Lane 2 to Lane 20 isolates.

Isolates of *Aeromonas spp.* have the *Aero* gene. The PCR results matched with Yogananth *et al.*, 2009 and Niamah AK, 2012. As indicated the presence of *Aero* gene in found in *Aeromonas spp.* which were isolated from water samples. *A. hydrophila, A. veronii, A. sobria, A. caviae* and *A.*

salmonicida subsp. Salmonicida showed presence of Aero gene. Whereas isolates like *A. euccrenophila, A.popoffii* and *A. trota;* the Aero gene was absent. This study proved presence *Aeromonas spp.* in water distribution system of Surat city. Thus the PCR assay used in this study proved to be a useful tool for the detection of virulent *Aeromonas spp.*by detecting aerolysin genes as genetic virulence markers. The molecular characterization of the isolates showing the presence of Aero gene were further proceeded for 16S rDNA sequencing.

Conclusion

From the study, different *Aeromonas spp.* were isolated from different water samples within the distribution systems. The seasonal variation was also observed. The isolates also have capacity to produce and live within Biofilm on the surface along with chlorine tolerance. The organisms might not be suceptible, but when they form biofilm it increases the ability of organism to cause disease. Thus formation of biofilm can contribute to the pathogenicity of the organism. Thus it serves to check the quality of water and for the purpose of public health. The quality of drinking water must be check for the presence of *Aeromonas* spp. in water and necessary treatment or process should be implemented in future prospect.

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